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Rio2p, an Evolutionarily Conserved, Low Abundant Protein Kinase Essential for Processing of 20 S Pre-rRNA in *Saccharomyces cerevisiae**

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Saccharomyces cerevisiae Rio2p (encoded by open reading frame *Ynl207w*) is an essential protein of unknown function that displays significant sequence similarity to Rio1p/Rrp10p. The latter was recently shown to be an evolutionarily conserved, predominantly cytoplasmic serine/threonine kinase whose presence is required for the final cleavage at site D that converts 20 S pre-rRNA into mature 18 S rRNA. A data base search identified homologs of Rio2p in a wide variety of eukaryotes and Archaea. Detailed sequence comparison and *in vitro* kinase assays using recombinant protein demonstrated that Rio2p defines a subfamily of protein kinases related to, but both structurally and functionally distinct from, the one defined by Rio1p. Failure to deplete Rio2p in cells containing a *GAL-rio2* gene and direct analysis of Rio2p levels by Western blotting indicated the protein to be low abundant. Using a *GAL-rio2* gene carrying a point mutation that reduces the kinase activity, we found that depletion of this mutant protein blocked production of 18 S rRNA due to inhibition of the cleavage of cytoplasmic 20 S pre-rRNA at site D. Production of the large subunit rRNAs was not affected. Thus, Rio2p is the second protein kinase that is essential for cleavage at site D and the first in which the processing defect can be linked to its enzymatic activity. Contrary to Rio1p/Rrp10p, however, Rio2p appears to be localized predominantly in the nucleus.

Like their counterparts in other eukaryotes, *Saccharomyces cerevisiae* ribosomes contain four species of rRNA: 5 S, 5.8 S, 18 S, and 25 S rRNAs. The genes encoding these rRNAs are organized on the yeast genome in 150–200 tandem repeats, each of which comprises two transcriptional units separated by non-transcribed spacers. One of these units consists of a 5 S rRNA gene, transcribed by RNA polymerase III. The other unit contains single genes for each of the mature 18 S, 5.8 S, and 25 S rRNAs that are separated by internal transcribed spacers 1 and 2, whereas external transcribed spacer regions are present at either end of the unit (see Fig. 1A). After transcription of this polycistronic unit by RNA polymerase I, the spacers are removed from the primary transcript via an ordered series of endo- and exonucleolytic cleavages (see Fig. 1B) (reviewed in

Refs. 1 and 2). The first detectable precursor species is 35 S pre-rRNA, which results from a cleavage at site B₀ in the 3'-external transcribed spacer by the yeast RNase III homolog Rnt1p (3, 4). Subsequent cleavage at sites A₀ and A₁ in the 5'-external transcribed spacer first gives rise to 33 S and then 32 S pre-rRNA. The latter is cleaved at site A₂ to produce separate 20 S and 27 S A₂ precursors for the small and large ribosomal subunit, respectively.

The majority (90%) of the 27 S A₂ precursor molecules are cleaved endonucleolytically at site A₃, followed by exonucleolytic trimming to B_{1S}. The remainder are processed endonucleolytically¹ at site B_{1L}. The resulting 27 S B_S and 27 S B_L precursors, whose 5'-ends are located 6 nucleotides apart, are then converted into 25 S rRNA and the "short" and "long" forms of 5.8 S rRNA, respectively, in the same manner (5–9). All of the above processing steps, as well as the concomitant assembly of most of the ribosomal proteins, take place in the nucleolus/nucleoplasm. However, the final step in 40 S subunit biogenesis in yeast occurs after export of the pre-40 S ribosomal subunit to the cytoplasm, where its 20 S pre-rRNA is processed at site D to produce the mature 18 S rRNA (10, 11). Pre-rRNA processing in other eukaryotic cells follows a similar pathway, except that maturation of 18 S rRNA is completed in the nucleus rather than the cytoplasm.

Although the pre-rRNA processing pathway itself is now well understood, many questions pertaining to its control and its integration with the assembly of the ribosomal proteins remain to be answered. Presently, >100 non-ribosomal proteins or *trans*-acting factors have been identified that are essential for ribosome biogenesis in *S. cerevisiae* (1, 12, 13). Recent advances in the purification and characterization of large (ribonucleo)-protein complexes have pushed the number of potential *trans*-acting factors even higher. Moreover, it has become clear that these factors form two largely independent, dynamic processing/assembly machineries for the small and large subunits, respectively (12, 14–17). Very little is known as yet about the exact function of any of the parts of these machineries or the manner in which they cooperate.

Virtually all of the *trans*-acting factors presently known to be involved in biogenesis of the yeast 40 S subunit are required for the formation of 20 S pre-rRNA by the early processing cleavages at sites A₀, A₁, and A₂. These factors assemble cotranscriptionally on the pre-rRNA in the nucleolus, together with some of the ribosomal proteins, to form the 80–90 S small subunit pre-ribosome and, at least for the most part, appear to dissociate prior to export of the pre-40 S particle to the cytoplasm (1, 12, 14, 15). However, a number of *trans*-acting factors remain associated or join the pre-40 S particle in the nucleo-

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¹ A. W. Faber, J. C. Vos, and H. A. Raué, unpublished data.

TABLE I
S. cerevisiae strains used in this study

Strain	Genotype	Ref.
Y22005	<i>Mata/α, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, ynl207w::kanMX4/YNL207w</i>	EUROSCARF
SC1413	<i>Mata, ade2, arg4, leu2-3,112, trp1-289, ura3-52, YNL207w::TAP-K.I.URA3</i>	EUROSCARF
SC1110	<i>Mata, ade2, arg4, leu2-3,112, trp1-289, ura3-52, YHR089c::TAP-K.I.URA3</i>	EUROSCARF
YTV101	<i>Mata, his3Δ1, leu2Δ0, ura3Δ0, ynl207w::kanMX4 (pWTR2)</i>	This work

plasm and accompany it upon export to the cytoplasm (18). One of these factors is Rrp10p, which is essential for the conversion of 20 S pre-rRNA into mature 18 S rRNA and was identified in a synthetic lethality screen with a mutant of Gar1p (11), one of the core proteins of box H/ACA small nucleolar ribonucleoproteins. Interestingly, Rrp10p, also known as Rio1p (19), was subsequently shown to be a member of a novel subfamily of serine/threonine protein kinases with family members in eukaryotes, Archaea, and prokaryotes (20–22). Homologs of Rio1p in other organisms have been implicated in cell cycle progression, and it has been reported that depletion of Rio1p in yeast causes arrest in the G₁ phase of the cell cycle (21, 22). The connection between these two functions of Rio1p/Rrp10p and its enzymatic activity remains unclear.

In yeast, the open reading frame *Ynl207w* encodes a protein called Rio2p that has 19% identity and 49% similarity to Rio1p/Rrp10p. The function of Rio2p in yeast cells is unknown, but obviously differs from that of Rio1p/Rrp10p as both proteins are essential. A systematic analysis of several hundreds of yeast protein complexes isolated by tandem-affinity purification (23) demonstrated the association of Rio2p on the one hand with a number of proteins required late in 40 S subunits biogenesis such as Trs1p, Rrp12p, and Dim1p and on the other hand with several proteins implicated in cell growth and maintenance (24, 25). In this study, we present evidence that Rio2p is a member of a second subfamily of protein kinases and, like Rio1p, is essential for cleavage at site D, the final processing step in the formation of mature 18 S rRNA.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The yeast strains and plasmids used in this study are listed in Tables I and II. All strains were grown on yeast nitrogen base medium (Difco) containing the relevant amino acids and either 2% (w/v) glucose or galactose. Plates also contained 2% agar (Bacto-agar, Difco). During determination of growth rates, cells were kept in logarithmic growth by regular dilution with prewarmed medium. All optical density measurements were performed at 660 nm on a Novaspec 550 spectrophotometer (Amersham Biosciences).

The basal strain for our analysis was constructed by first transforming the diploid Y22005 strain carrying a *rio2* gene inactivated by insertion of the kanamycin marker on one of its chromosomes (obtained from EUROSCARF) with the centromeric pWTR2(URA3) plasmid carrying a wild-type copy of the *RIO2* gene. The resulting transformants were sporulated using the method described on the EUROSCARF site,² except that our pre-sporulation plates contained 2× yeast nitrogen base medium and 5% (w/v) glucose and lacked uracil to select for the presence of the plasmid. After growing the dissected spores on YPD (1% yeast extract, 2% peptone, and 2% dextrose) plates, haploid transformants containing the inactivated *rio2* gene were identified by PCR. The selected haploid strain containing the *rio2::kan* gene was called YTV101. The pWTR2 plasmid present in the cells was replaced by the pGalR2(HIS3) plasmid or its mutant counterparts by plasmid shuffling.

Construction of Plasmid-encoded Wild-type and Mutant *RIO2* Genes and *RIO2* Fusion Genes—A plasmid-encoded copy of the *RIO2* gene under the control of its own promoter was constructed by amplifying the gene including 300 bp of its upstream sequence with the R2B1fw and R2E1rv primers (Table III). The resulting product was inserted between the *Bam*HI and *Eco*RI sites of pRS316(URA3) (26), resulting in plasmid pWTR2. The *RIO2* gene was fused to the *GAL1-10* promoter by

first amplifying it using the R2E1fw and R2S1rv primers (Table III). The resulting product was inserted into pTL26(HIS3) (27) between the *Eco*RI and *Xho*I sites to give the pGalR2 plasmid.

Point mutations (see Fig. 2) were introduced into the *RIO2* coding region by site-directed mutagenesis via the megabase primer method (28) using the unique *Ava*I, *Bst*EII, and *Hinc*II sites in the *RIO2* gene carried on the pGalR2 plasmid. The oligonucleotides used for mutagenesis are listed in Table III.

A GST³-*RIO2* fusion was constructed by amplification of the *RIO2* gene using the R2N1fw and R2E1rv primers (Table III). The former primer introduces an *Nco*I site at the ATG start codon of the *RIO2* coding region. The PCR product was inserted between the *Nco*I and *Eco*RI sites of plasmid pRP261, a derivative of pGEX1 (29) that fuses the *RIO2* and GST coding regions in-frame. This plasmid was transformed into the *Escherichia coli* Sure strain (Stratagene), and expression of the fusion protein was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside to an exponentially growing culture. After an additional 2 h at 30 °C, cells were collected and broken by sonication in lysis buffer containing 20% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 125 mM NaCl, 2.5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, and 0.1% Nonidet P-40. The extract was centrifuged for 15 min at 14,000 rpm in an Eppendorf centrifuge to remove the cell debris. Glutathione-Sepharose beads (400 μl; Amersham Biosciences) were added to the resulting supernatant. After incubation for 30 min at 4 °C, the beads were washed three times with lysis buffer. GST-Rio2p(D229A) was purified in the same manner from cells expressing a derivative plasmid in which the wild-type *RIO2* coding region had been replaced by the mutant version.

Biochemical Fractionation—20 OD₆₆₀ units of yeast cells harvested during mid-logarithmic growth were resuspended in buffer A (1 M sorbitol, 50 mM Tris-HCl (pH 8.0), and 10 mM MgCl₂) containing 30 mM DTT and incubated at room temperature for 15 min. The cells were then pelleted by centrifugation in an Eppendorf centrifuge for 1 min at 3000 rpm, resuspended in buffer A containing 3 mM DTT and 0.45 mg/ml zymolyase (Seikagaku), and incubated at 30 °C for 60 min. The cells were pelleted, washed with ice-cold buffer A, and resuspended in buffer B (15 mM KCl, 10 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.1 mM EDTA, and 3 mM DTT) using a homogenizer with a loose-fitting pestle. The cell suspension was incubated at 4 °C for 20 min and then broken with five strokes of a tight-fitting pestle. The resulting homogenate was separated into a cytoplasmic and a nuclear fraction by centrifugation for 20 min at 13,000 rpm in an Eppendorf centrifuge at 4 °C.

RNA and Protein Analysis—Total RNA was isolated by resuspending 20 OD₆₆₀ units of cells in 600 μl of buffer (10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% (w/v) SDS) and 600 μl of water-saturated phenol. After adding 100 μl of glass beads (diameter, 0.5–0.8 mm), the samples were heated at 65 °C for 1 h under continuous stirring. The aqueous phase was re-extracted with phenol and again with 600 μl of chloroform/isoamyl alcohol (24:1, v/v). RNA was precipitated by adding 40 μl of 3 M NaAc (pH 5.2) and 800 μl of EtOH and redissolved in RNase-free distilled water to a concentration of 0.4 A₂₆₀/μl. Northern analysis was performed as described previously using the various probes indicated in Fig. 1A (30).

Protein extracts for Western blotting were prepared by lysing the cells in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl, and 1 mM DTT with glass beads (diameter, 0.5–0.8 mm) by vortexing for 5 min. The extracts were subjected to SDS-PAGE on 12% minigels (Bio-Rad). After blotting onto nylon membrane (Protran, Schleicher & Schüll) and incubation with horse radish peroxidase-labeled antibodies against the protein A epitope, bound antibody was visualized using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate coloring reaction.

In Vitro Kinase Assays—The auto- and heterophosphorylating activ-

² Available at www.uni-frankfurt.de/fb15/mikro/euroscarf/index/html.

³ The abbreviations used are: GST, glutathione S-transferase; DTT, dithiothreitol; MBP, myelin basic protein; PAA, polyacrylamide; TAP, tandem affinity purification.

TABLE II
Plasmids used in this study

Plasmid		Ref.
pTL26	<i>HIS3, CEN/ARS, Amp, GAI1-10</i>	27
pRS316	<i>URA3, CEN/ARS, Amp</i>	26
pWTR2	Derived from pRS316 by insertion of <i>RIO2</i> under its own promoter between <i>Bam</i> HI and <i>Eco</i> RI sites	This work
pGalR2	Derived from pTL26 by insertion of the <i>GAL-rio2</i> gene between <i>Eco</i> RI and <i>Xho</i> I sites	This work
pGalR2(D229A)	Identical to pGalR2, but containing D229A mutation in <i>RIO2</i> coding region	This work
pGSTR2	Derivative of pGEX1 containing GST- <i>RIO2</i> fusion gene	This work
pGSTR2(D229A)	Identical to pGSTR2, but containing D229A mutation in <i>RIO2</i> coding region	This work

TABLE III
Sequences of oligonucleotides used in this work either as probes in Northern hybridization or as primers during construction of the various *RIO2* genes

All sequences are shown 5' → 3'. The position of the probes are indicated in Fig. 1A. Sequence alterations used in the introduction of point mutations are in boldface, and the nature of the mutation is indicated.

Probes	Sequence
1	GAAATCTCTCACCGTTTGAATACG
2	TGTTACCTCTGGGCCC
3	GGCCAGCAATTTCAAGTTAA
Primers	
R2E1fw	AAAAAGAATTCTCAGACTAGAATGAAATTGGATAC
R2E1rv	AAAAAGAATTCTGAACTTGAATTTGCGGC
R2B1fw	AAAAAGGATCCGGCTCACGTTGGTGAATGGCT
R2S1rv	AAAAAGTCGACAACAACCTGATTATTTGCGGC
Avalfw	CGAAGTCGTTCCAACCTCCCTTG
Bstefw	GGAGCTTATCGAGGGTTACCC
Bsterv	CTCCTCATTTGGGTAACCC
Hincrv	TCAGTTTCTTTTGAAGAAACGACG
VVI	CGCGTCATATAGTCGTTAAGGAGCTTATCGAGGGTTACCC (M189K)
VVK	CGCGTCATATAGTCGTTATTGAGCTTATCGAGGGTTACCC (M189I)
GRES	CGGTAACACTATTGGTGTGGTATTGAATCTGACATCTATAAAGTAAG (K105R)
GIES	CGGTAACACTATTGGTGTGGTATTGGAATCTGACATCTATAAAGTAAG (K105I)
GLIFCD	GCAAATAGCGGACTTATCTTTTGTGATTTTAATGAGTTTAATATT (H227F)
GLIYCD	GCAAATAGCGGACTTATCTATTGTGATTTTAATGAGTTTAATATT (H227Y)
GLIHCA	GCAAATAGCGGACTTATCCATTGTGCTTTTAATGAGTTTAATATT (D229A)

ities of recombinant GST-Rio2p were determined by incubating 5 μ l of Sepharose-glutathione-bound GST-Rio2p beads in 20 μ l of buffer containing 50 mM Tris-HCl (pH 7.2), 5 mM MgCl₂, and 1 μ Ci of [γ -³²P]ATP (10 mCi/ml; Amersham Biosciences) either with or without 5 mM MnCl₂. To determine the ability of Rio2p to heterophosphorylate a substrate, 4 μ g of myelin basic protein (MBP; Sigma) was added to the reaction mixture. The samples were analyzed by electrophoresis on a 12% SDS-PAA gel, followed by autoradiography. The amount of GST-Rio2p present in the reaction mixture was assayed by staining with Coomassie Brilliant Blue

RESULTS

Rio2p Is a Member of a Distinct Subfamily of Putative Protein Kinases—The *S. cerevisiae* open reading frame *Ynl207w* encodes a protein, designated Rio2p (19), that shows significant structural similarity to Rio1p/Rrp10p. The latter was found to be essential for endonucleolytic cleavage at site D, the final step in the formation of mature 18 S rRNA (11). Subsequently, it was characterized as a predominantly cytoplasmic serine/threonine protein kinase conserved from bacteria to man with a role in cell cycle progression (20–22).

Fig. 2 shows the results of an alignment of Rio1p/Rrp10p and Rio2p, demonstrating that the sequence similarities between the two proteins are predominantly confined to three regions indicated as I, II, and III.⁴ Regions I and III contain the putative ATP-binding and kinase domains, respectively (22). This result suggests that Rio2p also is a protein kinase, but may belong to a subfamily distinct from the one defined by Rio1p/Rrp10p.

Using the Rio2p sequence as our starting point, we searched the NCBI Protein Database for homologs of Rio2p in other organisms and identified a number of such homologs in both

eukaryotes and Archaea. Alignment of these homologs revealed a pattern of sequence conservation that is distinct from the one between Rio2p and Rio1p (Fig. 2). First, the N-terminal regions of the various Rio2 proteins show marked similarity, but differ considerably from the corresponding region in Rio1p, which is not significantly conserved in the latter subfamily (20, 22). Second, within the regions that are conserved between Rio1p and Rio2p, conservation within each of the two subfamilies is higher than between the two subfamilies (Fig. 2). From these data, we conclude that Rio2p defines a putative protein kinase subfamily that is related to, but distinct from, the one founded by Rio1p/Rrp10p. Phylogenetic analysis (Fig. 3) adds further support to this conclusion. This analysis clearly shows an early division between Rio1p- and Rio2p-like proteins. Furthermore, a small number of (archaeo)bacterial Rio-like proteins seem to form a third subfamily.

Interestingly, our data base search did not identify any eubacterial members of the Rio2p subfamily. Thus, whereas eukaryotes and Archaea have at least one gene member in both the *RIO1* and *RIO2* subfamily, the latter seems to be absent from the eubacterial kingdom. This suggests that the two types of subfamily originated from duplication of an ancestral gene that occurred before the split between Archaea and eukaryotes. Because Rio1p and Rio2p are both essential in yeast, the two genes gained different functions.

Low Level Expression of Rio2p Is Sufficient to Support Normal Growth—To study the role of Rio2p in yeast in more detail, we constructed a mutant strain that conditionally expresses the protein. As the starting point, we used the diploid strain Y22005 obtained from EUROSCARF. This strain contains a *rio2* gene that has been disrupted by insertion of the kanamycin gene on one of its chromosomes.

⁴ A detailed alignment is available upon request.

FIG. 1. **Pre-rRNA processing in *S. cerevisiae*.** A, structure of the rDNA repeat, including the processing sites used for formation of the mature 18 S, 5.8 S, and 25 S rRNA species. The positions of the probes employed for Northern hybridization (Table III) are indicated. B, the pre-rRNA processing pathway. *Pol*, RNA polymerase.

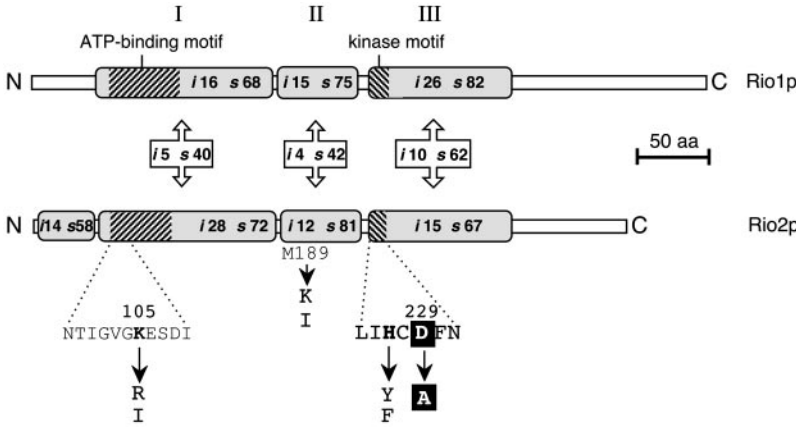
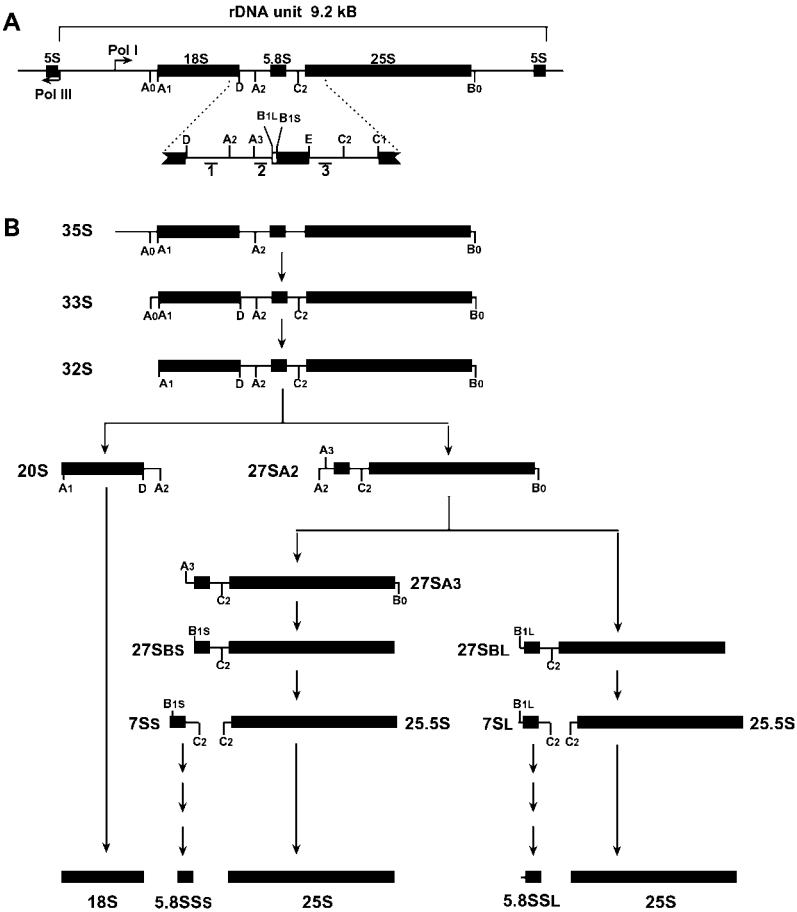
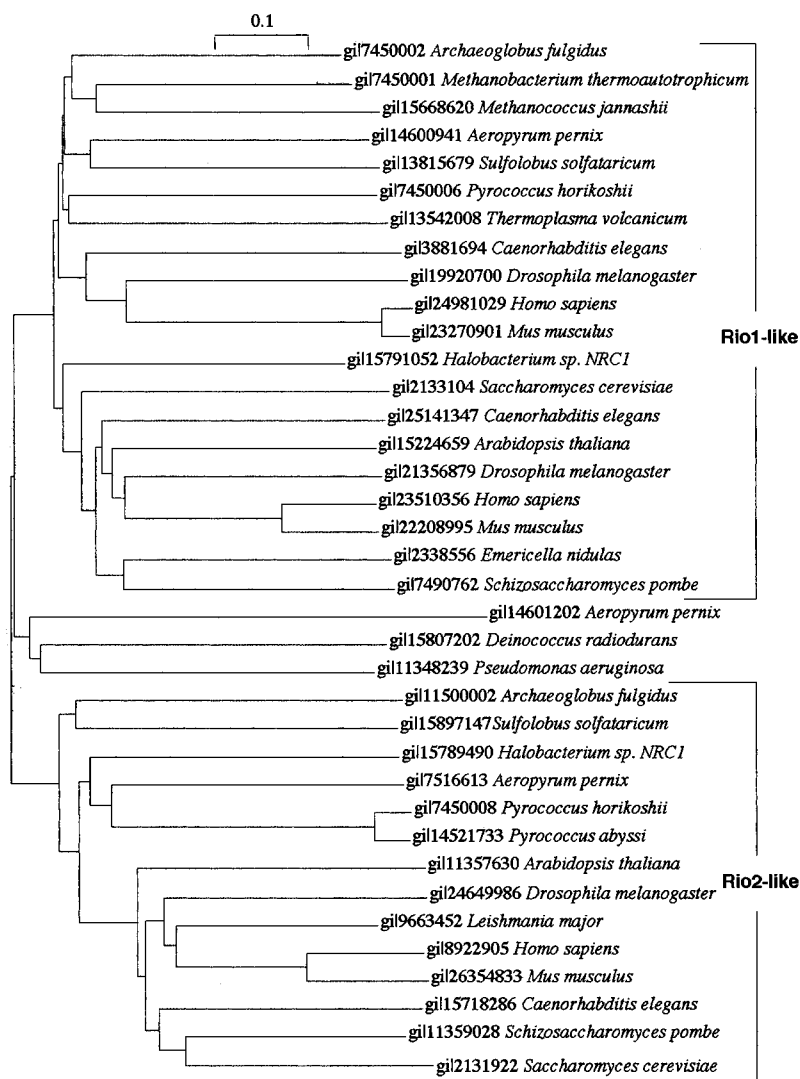


FIG. 2. **Sequence conservation within and between the subfamilies of homologous proteins defined by Rio1p and Rio2p.** Sequences for Rio1p and its subfamily were taken from Ref. 22. Members of the Rio2p subfamily were identified by searching the NCBI Protein Database with the Rio2p sequence using the ClustalW algorithm. Conserved regions are shaded. The numbers within these shaded regions indicate the percentage of sequence identity (*i*) and sequence similarity (*s*) within the pertinent subfamily. The numbers within the double-headed boxes indicate the percentage of sequence identity and similarity between the two subfamilies in the particular region. The positions and nature of the mutations introduced into Rio2p are indicated. aa, amino acids.

Diploid Y22005 cells were transformed with the centromeric pWTR2(URA3) plasmid carrying a wild-type *RIO2* gene under the control of its authentic promoter. Transformants were selected on plates lacking uracil; and after sporulation, tetrads were dissected and spotted on YPD plates. Transformation of Y22005 with an empty vector showed the expected 2:2 segregation of viable and nonviable spores. PCR analysis demonstrated that none of the viable spores contained the disrupted *rio2* gene, confirming that *RIO2* is an essential gene as previously reported (31). In contrast, sporulation of Y22005 cells carrying the pWTR2 plasmid in most cases resulted in four viable spores. The resulting haploid transformants were tested for G414 resistance to ascertain the presence of the disrupted chromosomal *rio2* gene as well as for their ability to grow on plates lacking uracil to ensure retention of the plasmid. As a final check, selected cells were analyzed by PCR to ascertain

that no recombination had taken place between the plasmid and the genome. This resulted in strain YTV101(pWTR2) (*rio2*-null), which was found to grow normally. To determine the effect of depletion of Rio2p, we replaced the pWTR2 plasmid with plasmid pGalR2(HIS3), which contains the wild-type *RIO2* gene under the control of the repressible *GAL1-10* promoter, by plasmid shuffling and determined the effect of a shift from galactose to glucose on growth of the YTV101(pGalR2) transformants. To our surprise, we found no significant change in growth rate after the shift compared with the wild-type control, for which we took cells grown from viable haploid spores of the Y22005 strain that had been transformed with an empty vector. Even after culturing the pGalR2-transformed YTV101 cells for several days on glucose-based medium, normal growth was maintained (*cf.* Fig. 4) (data not shown). This suggests that the small amount of protein resulting from leak-

FIG. 3. **Phylogenetic tree for the Rio1p- and Rio2p-related proteins.** A phylogenetic tree for the various Rio1p- and Rio2p-related sequences extracted from the data base was constructed using the ClustalW program. Accession numbers for the various sequences in the NCBI Protein Database are given.



iness of the *GAL1-10* promoter under repressive conditions is sufficient to support normal growth.

Mutation of Conserved Residues in Rio2p—To circumvent the problem posed by the requirement for only low level expression of Rio2p, we decided to introduce a number of point mutations at strongly conserved positions in the protein that might either cause temperature sensitivity or reduce its activity. We chose primarily to target conserved amino acids located within the putative ATP-binding motif or the kinase site as indicated in Fig. 2. The mutant genes were constructed by site-directed mutagenesis (see “Experimental Procedures”) and used to replace the wild-type *RIO2* coding region in the pGalR2 plasmid. The resulting plasmids were then transformed into YTV101(pWTR2) cells, and transformants were selected on plates lacking uracil and histidine and containing glucose as the carbon source to inhibit expression of the Rio2p mutant. Selected cells were tested for their ability to lose the pWTR2 plasmid by streaking them on plates containing 5-fluoroorotic acid and either galactose or glucose as the carbon source. With a single exception, all of the transformants were able to grow on 5-fluoroorotic acid plates containing either carbon source, indicating that the Rio2p mutants are functional and can support growth even when transcription of the encoding gene is repressed. The exception was the mutant in which the conserved Asp at position 229 in the putative kinase domain had been changed to Ala (Fig. 2). Cells expressing Rio2p containing the D229A mutation were able to grow on galactose (but not glu-

cose)-containing plates in the presence of 5-fluoroorotic acid. This suggests that the D229A mutation does not abolish the activity of the protein, but does reduce this activity to such an extent that low level expression of the repressed *GAL*-driven mutant *rio2* gene is insufficient for the need of the cell.

None of the Rio2p mutants displayed either temperature or cold sensitivity or a dominant-negative growth phenotype (data not shown). Therefore, we chose the D229A mutant to investigate the function of Rio2p further.

Strain YTV101 containing either pGalR2 or pGalR2(D229A) was grown on galactose and then shifted to glucose. As shown in Fig. 4, the strain expressing wild-type Rio2p kept growing with a doubling time of ~2.5 h even 48 h after the shift, confirming our conclusion that low level expression of Rio2p from the repressed *GAL-rio2* gene is sufficient to support normal cell growth. In contrast, the growth rate of the pGalR2(D229A) transformant, although initially identical to its wild-type counterpart, started to decrease strongly ~20 h after the shift. Equal amounts of cells were taken from both cultures 48 h after their transfer to glucose-containing medium, and a series of consecutive 10-fold dilutions was spotted on a yeast/peptone/galactose plate (Fig. 4, *inset*). No difference in growth could be observed between the cells expressing either the wild-type or mutant protein, indicating that depletion of Rio2p(D229A) causes growth arrest of the cells, but, at least initially, is not lethal. In contrast to cells depleted of Rio1p, however (22), YTV101 cells containing the *GAL-rio2*(D229A)

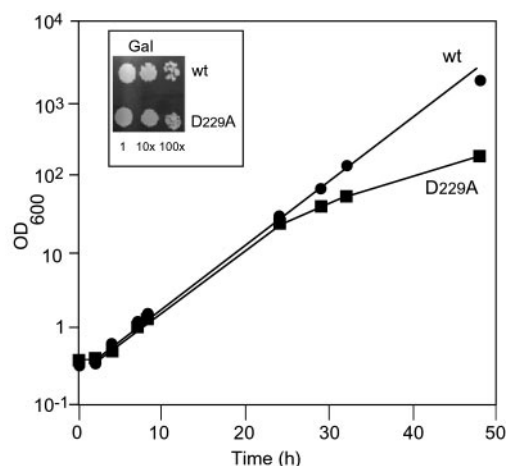


FIG. 4. Growth analysis of cells expressing a plasmid-encoded copy of the wild-type *RIO2* or *rio2*(D229A) mutant gene under the control of the *GAL* promoter. YTV101 cells transformed with either the pGalR2 or pGalR2(D229A) plasmid were first grown on medium containing galactose and shifted to glucose-containing medium at time 0. Growth was followed by determining the OD₆₀₀, and cells were kept in exponential growth by diluting the culture at regular intervals. ●, wild type (*wt*); ■, D229A mutant. The inset shows the results of growth on a galactose-containing plate of a dilution series of wild-type and mutant cells collected 48 h after the shift to glucose.

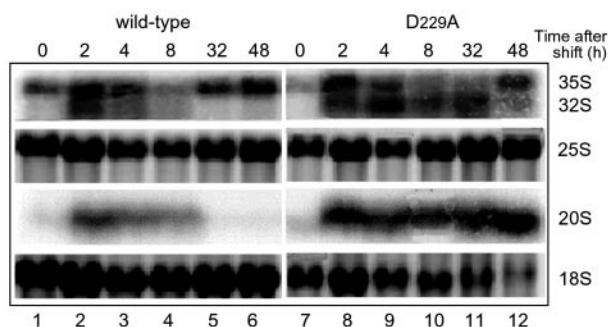


FIG. 5. Effect of Rio2p(D229A) depletion on pre-rRNA processing. YTV101 cells transformed with either the pGalR2 (*lanes 1–6*) or pGalR2(D229A) (*lanes 7–12*) plasmid were shifted from galactose- to glucose-containing medium, and samples were taken directly before and at different times after the shift. Total RNA was isolated from these samples and analyzed by Northern hybridization using the different probes indicated in Fig. 1A and Table III.

gene did not show any obvious morphological abnormalities even after prolonged culturing on glucose (data not shown).

Rio2p Is Required for Processing of 20 S Pre-rRNA into Mature 18 S rRNA—Because Rio2p has been found to be physically associated with proteins implicated in late steps in the formation of 40 S ribosomal subunits (24), we wanted to analyze the effect of depletion of Rio2p(D229A) on ribosome biogenesis. To that end, total RNA was isolated from pGalR2- and pGalR2(D229A)-transformed YTV101 cells harvested at different times after a shift from galactose to glucose. The RNA samples were subjected to Northern analysis using various probes to determine the levels of the different mature and precursor rRNA species. Whereas there was no effect on either 25 S or 18 S rRNA production in the pGalR2 transformants (Fig. 5, *lanes 1–6*), depletion of Rio2p(D229A) resulted in a progressive reduction in the level of 18 S rRNA starting ~20–24 h after the shift, and the amount of 25 S rRNA remained constant over a 48-h period (*lanes 7–12*). Furthermore, the mutant strain showed a strong accumulation of 20 S pre-rRNA as well as some increase in the level of 32 S pre-rRNA. Thus, we conclude that Rio2p has a major role late in 40 S

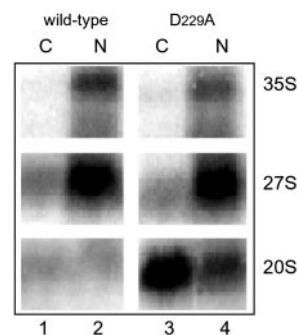


FIG. 6. Subcellular localization of 20 S pre-rRNA in cells depleted of Rio2p(D229A). YTV101 cells transformed with either the pGalR2 (*lanes 1 and 2*) or pGalR2(D229A) (*lanes 3 and 4*) plasmid were shifted from galactose- to glucose-containing medium. Cells were harvested 24 h after the shift, and nuclear (N) and cytoplasmic (C) fractions were prepared. Total RNA was isolated from each of these fractions, and the levels of 20 S pre-rRNA were analyzed by Northern hybridization using probe 1 (indicated in Fig. 1A and Table III). As a control, we also analyzed the levels of the 35 S and 27 S precursors using probes 1 and 3, respectively.

subunit biogenesis, either in the nuclear export of the pre-40 S particle or in the final processing cleavage at site D. The increase in the level of the 32 S precursor suggests that the absence of Rio2p may also delay the early cleavage at site A₂. This delay did not have detectable consequences for 25 S rRNA production, however (Fig. 5).

To define the role of Rio2p in 40 S subunit biogenesis more precisely, we analyzed the subcellular distribution of the 20 S precursor in the Rio2p(D229A)-depleted cells using a biochemical approach. Extracts from YTV101 cells expressing either the plasmid-encoded wild-type or mutant *GAL::rio2* gene were prepared after growth on glucose for 24 h by carefully breaking the cells, followed by separation into a nuclear and a cytoplasmic fraction using differential centrifugation. The level of 20 S pre-rRNA in each of the fractions was then determined by Northern analysis. As shown in Fig. 6, cells containing the *GAL*-driven wild-type *RIO2* gene contained roughly equal, low amounts of 20 S pre-rRNA in both fractions (*lanes 1 and 2*). In contrast, in cells that depend upon the *GAL*-driven *rio2*(D229A) gene, a much higher level 20 S pre-rRNA was found, most of which was present in the cytoplasmic fraction (*lanes 3 and 4*). Both types of cells showed a very similar distribution of 35 S and 27 S pre-rRNAs, with only a small percentage present in the cytoplasmic fraction. Thus, the high level of 20 S pre-rRNA found in the cytoplasmic fraction from the mutant cells is not due to excessive leakage from damaged nuclei. We conclude that, in cells depleted of Rio2p(D229A), the 20 S pre-rRNA does get exported from the nucleus, but fails to be processed further.

Rio2p Is a Protein Kinase—To determine whether Rio2p is indeed able to function as a protein kinase and whether the D229A mutation affects this activity, we fused wild-type Rio2p as well as Rio2p(D229A) N-terminally to GST, expressed the fusion proteins in *E. coli*, and purified them from cell extracts using glutathione-Sepharose beads (see “Experimental Procedures”). When the purified material was analyzed on an SDS-polyacrylamide gel, in both cases, a band was visible at ~80 kDa, the expected molecular mass of GST-Rio2p (Fig. 7, *lanes 2 and 3*), and contamination with other proteins was minor. Material purified from the control cells transformed with the same vector containing only the GST gene produced a strong band corresponding to GST, but no signal at the position of GST-Rio2p (*lane 1*).

First, we analyzed whether Rio2p, like Rio1p (21, 22), is capable of autophosphorylation by incubating glutathione-

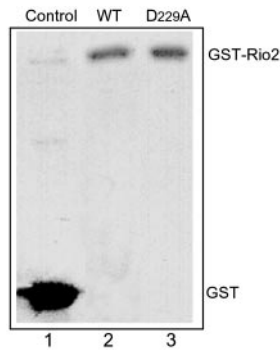


FIG. 7. Purification of GST-Rio2p and GST-Rio2p(D229A). GST-Rio2p and GST-Rio2p(D229A) were expressed in *E. coli* and purified by affinity chromatography using glutathione-Sepharose beads. Bound material was analyzed by SDS-PAGE gel electrophoresis, and protein was visualized by staining with Coomassie Brilliant Blue. Lane 1, protein purified from cells transformed with a plasmid encoding GST; lane 2, protein from cells expressing GST-Rio2p (wild-type (WT)); lane 3, protein from cells expressing GST-Rio2p(D229A).

bound GST-Rio2p and GST-Rio2p(D229A) with [γ - 32 P]ATP in a Tris/MgCl₂ buffer, followed by separation of the labeled products by SDS-PAGE (Fig. 8A). Both wild-type Rio2p and Rio2p(D229A) were indeed able to phosphorylate themselves, but the mutant protein was considerably less efficient (*cf.* lanes 2 and 3). No labeled products were visible in the control (lane 1), demonstrating that the kinase activity is due to the recombinant protein. Furthermore, in this buffer, neither the wild-type nor mutant protein proved to be able to phosphorylate MBP, and the addition of MBP did not affect the rate or extent of autophosphorylation (data not shown). Therefore, we also determined the kinase activity of wild-type Rio2p and Rio2p(D229A) in the presence of 5 mM MnCl₂, which has been shown to improve the efficiency of both auto- and heterophosphorylation by Rio1p (21). As shown in Fig. 8B, the addition of MnCl₂ did not significantly affect the rate of autophosphorylation by Rio2p (Fig. 8, *cf.* A and B, lanes 2 and 3). However, in the presence of MnCl₂, substantial phosphorylation by wild-type Rio2p of the heterologous substrate MBP could be observed (Fig. 8B, lane 1). The D229A mutant protein was also capable of phosphorylating MBP under these conditions, but this activity was reduced to the same extent as its autophosphorylating activity (lane 2). These results demonstrate that Rio2p is a protein kinase and that the D229A mutation significantly impairs its enzymatic activity.

As a control, we carried out similar experiments with various other Rio2p mutants (Fig. 2). Fig. 8 (A and B, lanes 3) shows the results for the mutant in which His²²⁷, which is absolutely conserved in both the Rio2p and Rio1p/Rp10p subfamilies, was changed into Phe (Fig. 2). Clearly, both the auto- and heterophosphorylating activities of Rio2p were only marginally (if at all) affected by this non-conservative replacement, thus strengthening the argument that the role of Rio2p in pre-rRNA processing is linked to its enzymatic activity. Attempts to test the kinase activity of the K105I mutation in the kinase domain were unsuccessful because we were unable to express this mutant protein in *E. coli*. However, the absence of a detectable growth defect upon depletion of Rio2p(K105I) strongly indicates that, like the H227F mutation, this non-conservative replacement does not significantly affect the enzymatic activity.

Rio2p Is Expressed at a Low Level and Is Predominantly Nuclear—To determine the subcellular localization of Rio2p, we used strain SC1413 (obtained from EUROSCARF), which contains a *RIO2* gene controlled by its own promoter that encodes a TAP-tagged version of the protein. Our initial at-

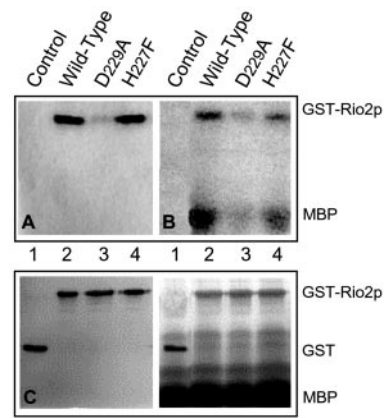


FIG. 8. In vitro kinase assay of recombinant wild-type Rio2p and its mutants. Glutathione-Sepharose beads containing similar amounts of GST-Rio2p (wild-type), GST-Rio2p(D229A), or GST(H227F) were incubated for 30 min under different buffer conditions in the presence of [γ - 32 P]ATP, and the labeled products were analyzed by SDS-PAGE gel electrophoresis. A, incubation in Tris-HCl (pH 7.2) containing 5 mM MgCl₂; B, incubation in the same buffer, but containing, in addition, 5 mM MnCl₂ as well as 4 μ g of MBP as heterologous substrate; C, SDS-PAGE gel electrophoretic analysis of an aliquot of the incubation mixture to compare the amount of recombinant protein present in the assay mixture. Lanes 1, protein from *E. coli* cells expressing only GST; lanes 2, protein from cells expressing GST-Rio2p (wild-type); lanes 3, protein from cells expressing GST-Rio2p(D229A); lanes 4, protein from cells expressing GST-Rio2p(H227F).

tempts to localize TAP-Rio2p using indirect immunofluorescence did not yield any conclusive data, probably due to the very low expression level of the protein (see below). We therefore again turned to biochemical fractionation. Total protein was isolated from both the nuclear and cytoplasmic fractions prepared from exponentially growing SC1413 cells and separated on a 10% SDS-PAGE gel. Tagged Rio2p was then visualized by Western blotting using an antibody against the ProtA moiety of the epitope tag. As a control, we performed the same experiment with strain SC1110 expressing TAP-tagged Gar1p, a protein known to be localized to the nucleolus (32, 33). As shown in Fig. 9A, TAP-tagged Gar1p could be readily detected and was indeed found predominantly in the nuclear fraction (lane 1), although there was some contamination of the cytoplasmic fraction (lane 2). Analysis of the nuclear and cytoplasmic fractions from strain SC1413 containing the same amount of total protein did produce a signal for TAP-Rio2p in the nuclear fraction, which, however, was much weaker than that obtained for TAP-Gar1p (Fig. 9B, lane 3). No Rio2p signal was detectable in the cytoplasmic fraction (lane 4) even upon more prolonged exposure (data not shown). From these data, we conclude 1) that Rio2p is a predominantly nuclear protein and 2) that the cellular level of Rio2p expressed from its authentic promoter is at least 2 orders of magnitude lower than that of Gar1p. The latter conclusion is in agreement with our failure to deplete Rio2p below the level required for normal growth in cells containing a *GAL-rio2* gene after a shift from galactose to glucose.

DISCUSSION

Over the past 3 decades, biochemical and genetic screens have identified many non-ribosomal factors essential for the formation of ribosomes in the yeast *S. cerevisiae* (2, 13). The majority possess structural (and thus presumably functional) homologs in other organisms ranging from *Caenorhabditis elegans* to human, underscoring the strong evolutionary conservation of the mechanics of eukaryotic ribosome biogenesis. Recent experiments have made it clear that these *trans*-acting factors are part of two largely independent, dynamic machin-

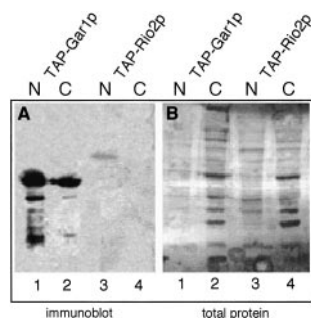


FIG. 9. **Subcellular localization of Rio2p.** Strains SC1110 and SC1413 expressing TAP-tagged versions of Gar1p (lanes 1 and 2) and Rio2p (lanes 3 and 4), respectively, were grown to mid-exponential phase and harvested by centrifugation. Extracts were prepared and separated into cytoplasmic (C) and nuclear (N) fractions by differential centrifugation. The fractions were analyzed by SDS-PAGE gel electrophoresis to compare their protein contents (B) and by Western blotting using an antibody against the ProtA moiety of the TAP tag to determine the level of the tagged protein (A).

eries that assemble on the pre-rRNA and govern its processing and ordered assembly with the ribosomal proteins into the small and large subunits, respectively (1, 12, 14, 15, 17). Functional characterization of these factors is still in its infancy; but, apart from the exo- and endonucleases obviously required for the removal of the spacer regions, they are known to include RNA helicases, modifying enzymes (methylases and pseudouridylylase), RNA-binding proteins, GTPases/ATPases, ribosomal protein assembly chaperones, and factors involved in intranuclear transport and nuclear export (reviewed in Refs. 1, 12, 13, and 17). Recently, protein kinases were added to this list by the finding that Rio1p/Rrp10p, a predominantly cytoplasmic serine/threonine protein kinase (21, 22), is essential for the endonucleolytic cleavage of 20 S pre-rRNA at site D that produces mature 18 S rRNA (11). The data reported in this study identify a second protein kinase, Rio2p, required for this final step in 18 S rRNA formation and also indicate a link between its enzymatic activity and 20 S pre-rRNA processing.

Rio2p shows significant sequence similarity to Rio1p/Rrp10p and, in particular, contains the structural motifs implicated in ATP-binding and kinase activities. However, detailed structural comparison indicated that the two proteins belong to separate subfamilies (Figs. 2 and 3). That founded by Rio1p/Rrp10p probably is the older one, as members of this subfamily are found in eubacteria (20), whereas members of the Rio2p subfamily are not. Our database search also identified Rio-like proteins in several (archae) bacterial species that have diverged considerably from both Rio1p/Rrp10p and Rio2p, but do group together, thus forming a third subfamily (Fig. 4). In yeast, Rio1p/Rrp10p and Rio2p have non-overlapping functions because both genes are essential (this study and Ref. 22). Transcriptome analysis (34) indicated that both proteins are expressed at low levels (~ 1 molecule of mRNA/cell). However, our data show that the requirement for Rio2p is distinctly lower than that for Rio1p/Rrp10p. Whereas the latter protein is functionally depleted by repressing transcription of a *GAL*-driven gene (11, 22), *GAL::rio2* cells grow at approximately wild-type rates on both liquid and solid media containing glucose (cf. Fig. 4). Apparently, the leakiness of the *GAL* promoter allows production of sufficient Rio2p to adequately cover cellular demand even under repressive conditions. The Western blot experiment shown in Fig. 9 further supports the conclusion that Rio2p is a low abundant protein, expressed at a level ~ 100 -fold below that of the small nucleolar ribonucleoprotein component Gar1p.

The lack of a detectable phenotype in *GAL::rio2* cells necessitated the construction of a conditional Rio2p mutant. To that end, we mutated absolutely conserved amino acids in the ATP-

binding and kinase motifs as well as in the central region II of a *GAL::rio2* gene (Fig. 2). Six of the seven mutants constructed remained able to support normal growth, even on glucose, when introduced into a *rio2*-null strain. This included both a conservative (Lys \rightarrow Arg) and a non-conservative (Lys \rightarrow Ile) replacement at position 105 in the ATP-binding motif, replacement of Met¹⁸⁹ with either Lys or Ile in the central region, and replacement of His²²⁷ with Tyr or Phe in the kinase domain. Thus, none of these residues, which are also conserved in Rio1p/Rrp10p, are essential for the function of Rio2p. However, the seventh mutation, a non-conservative replacement of Asp²²⁹ with Ala, did show a useful phenotype: whereas the mutant protein remained able to support normal growth on galactose, after a shift to glucose, a progressive decrease in the growth rate occurred starting ~ 20 h after the shift (Fig. 4). Northern experiments demonstrated a concomitant reduction in the level of 18 S rRNA, whereas production of the large subunit rRNAs remained unaffected (Fig. 5). The reduced level of 18 S rRNA is due to a block in cleavage of its immediate precursor at site D rather than inhibition of nuclear export of 43 S pre-ribosomes because 20 S pre-rRNA accumulated predominantly in the cytoplasm (Fig. 6). Thus, like Rio1p/Rrp10p, Rio2p is specifically required for the final cytoplasmic processing step in 18 S rRNA formation, but at a substantially lower level. So far, the only other example of a *trans*-acting factor that cannot be functionally depleted when expressed from a *GAL*-driven gene is Ngl2p, a nuclease required for the last (although nonessential) step in 3'-end formation of 5.8 S rRNA (6).

In apparent contradiction to the involvement of Rio2p in cytoplasmic processing of 20 S pre-rRNA, biochemical fractionation experiments clearly indicated that the protein is predominantly located in the nucleus (Fig. 9). This finding is in agreement with the presence of Rio2p in a complex containing a number of different *trans*-acting factors, including Dim1p, Rrp12p, and Tsr1p (24), which are also predominantly nuclear (or even nucleolar) proteins involved in 40 S subunit biogenesis (12, 35, 36). However, Dim1p is clearly part of both the nucleolar and cytoplasmic 40 S subunit processing/assembly machineries (35, 37), and the other components of the complex also appear to accompany the 43 S pre-ribosome upon its export from the nucleus (12, 18). A similar shuttling could reconcile the requirement of Rio2p for cytoplasmic processing of 20 S pre-rRNA with its predominant nuclear localization. In fact, evidence for such shuttling of Rio2p was reported while this manuscript was being revised (18, 38). However, the singularly low level cellular demand for Rio2p suggests that it acts catalytically rather than being a stoichiometric component of the 43 S pre-ribosome.

The *in vitro* assays using affinity-purified recombinant protein convincingly demonstrated that Rio2p is a protein kinase capable of both auto- and heterophosphorylation (Fig. 8). However, the latter activity required the inclusion of Mn²⁺ ions in the incubation buffer. The significant decrease in both auto- and heterophosphorylation caused by the D229A mutation (Fig. 8) indicates a link between the enzymatic activity of Rio2p and its role in 20 S pre-rRNA processing. This link is further strengthened by the fact that the H227F mutation in the kinase domain of Rio2p did not significantly affect the kinase activity. We cannot formally exclude the possibility that the processing phenotype of the D229A mutant is due to a lower expression level of the mutant protein rather than a reduced kinase activity. However, lower expression would most likely be the result of a higher rate of turnover of the mutant protein relative to its wild-type counterpart. We deem this possibility to be rather remote, as mutation of the closely neighboring His²²⁷ residue did not affect the ability of Rio2p to support

normal growth even when expressed from a *GAL* promoter under repressive conditions, indicating that this mutant is expressed at a normal level.

The physical association of Rio2p with proteins involved in chromatin remodeling (Snf2p, Snf5p, and Swi3p) and cell cycle progression (Bem2p and Hrr25p) (24, 25, 39) suggests that Rio2p is a multifunctional protein with a role in both ribosome biogenesis and cell proliferation. Cells depleted of Rio2p(D229A) could resume growth when transferred to galactose-based medium (Fig. 4), indicating that they have not reached a terminal phenotype. However, the Rio2p-depleted cells did not show any morphological features diagnostic of arrest at a particular stage of the cell cycle, in contrast to Rio1p/Rrp10p-deficient cells, the cytological analysis of which indicated arrest upon either entrance into S phase or exit from mitosis (22).

Although the negative effect of the D229A mutation in Rio2p on both its enzymatic activity and 20 S pre-rRNA processing indicates a link between its enzymatic activity and its role in cleavage of 20 S pre-rRNA at site D, the apparent multifunctional character of Rio2p raises the question of whether the pre-rRNA processing defect might be a secondary (rather than a primary) effect. The physical association of Rio2p with the processing/assembly complex involved in the late steps of 40 S subunit biogenesis (18, 24) strongly argues in favor of the latter. The target of this enzymatic activity (either Rio2p itself or another component of the 43 S pre-ribosome) remains to be identified.

Physical interaction of Rio1p/Rrp10p with components of the small subunit processing/assembly machinery has not been demonstrated directly. However, Rio1p/Rrp10p was found to co-sediment with 20 S pre-rRNA, suggesting that it is associated with the 43 S pre-ribosome and also may act catalytically (11). The effect of mutations that reduce its kinase activity and cause growth arrest (22) on 20 S pre-rRNA processing remains to be determined.

There are several precedents for proteins that function in both cell cycle progression and pre-rRNA processing. Nop7p (also known as Yhp1p) is required for exonucleolytic processing of 27 S A₃ pre-rRNA, whereas mutant alleles of the gene or depletion of the protein causes growth arrest in G₁ or G₂ (40–42). A similar dual function has been reported for its vertebrate homolog Pescadillo (43). Erb1p is required for cleavage at site C₂ within internal transcribed spacer 2 (44) and has a mammalian homolog, Bop1p, mutants of which cause a strong inhibition of pre-rRNA processing as well as a p53-dependent cell cycle arrest (45). A third example is Ytm1p, which is also involved in 60 S subunit biogenesis (16) and is essential for G₁/S transition (46). All three are found in 66 S pre-ribosomes (12) as well as in association with proteins involved in recognition of the origin of replication, cell cycle regulation, and chromatin remodeling (41). Thus, they are prime candidates for coordinating DNA replication and ribosome (in particular, 60 S subunit) biogenesis with cell proliferation. We suggest that Rio1p/Rrp10p and Rio2p have a similar role at the level of 40 S subunit biogenesis.

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